Materials and Methods Relating to the Modulation of T cell response to soluble antigen

5   **Field of the invention**

The present invention relates to materials and methods for improving vaccination strategies. Particularly, but not exclusively, the present invention relates to the use of an

10   activator of NKT cells as an adjuvant to enhance T-cell responses to soluble antigen.

Background of the invention

15   A significant challenge for the immune system is to be able to distinguish between harmful and benign antigenic stimuli in order to avoid inappropriate adaptive responses. Evidence suggests that modification in the function of antigen-presenting cells (APC), specifically dendritic cells (DC),

20   plays a critical role in this process (1). DC reside in peripheral tissues in an immature state defined by significant capacity for antigen uptake and low expression of MHC and costimulatory molecules. Upon exposure to inflammatory stimuli, DC undergo a maturation process involving migration

25   to lymphoid sites, down-regulation in rate of antigen uptake and increase in levels of MHC and costimulatory molecules. These changes enable DC to present antigens to naive CD4+ and CD8+ T cells and thereby promote adaptive immune responses (2).

30   The potency of DC as APC for naïve T cells can also be modulated directly by microbial stimuli via pattern recognition receptors, of which toll-like receptors are the prime examples (3), and also by signals such as CD40L (4, 5), RANKL (6) and FasL (7) mediated by T cells themselves. With

35   respect to the latter T 'helper' function, studies have shown that CD4+ T cells can activate DC specifically through signals

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mediated via CD40, inducing further upregulation of surface
molecules involved in T cell stimulation and inducing the
release of IL-12 (5). This 'conditioning' of DC is
particularly important for the generation of CTL from naïve

5   CD8$^+$ T cell precursors (8-10). It has been reported that
maturation of DC in the absence of CD40 signalling can
diminish the ability of these cells to release IL-12 (11), and
therefore potentially weakens their stimulatory capacity for
CTL. T cell help is therefore most likely to be effective if

10   it is achieved through interaction with immature DC early in
the evolution of a T cell-mediated response.

Exogenous protein antigens can be taken up by APC and
processed into peptides for presentation on the cell surface

15   by MHC class II molecules to CD4$^+$ T cells. This has been
referred to as the "exogenous" pathway of antigen
presentation. In contrast, internal antigens are processed
and presented by MHC class I molecules to CD8$^+$ T cells; the so-
called "endogenous" pathway of antigen presentation (18, 19).

20   It is now becoming clear that some APC, including subsets of
DC, are capable presenting peptides derived from exogenous
antigens on MHC class I molecules, resulting in the
stimulation of CD8$^+$ T cell responses (20-22). This would be a
desirable outcome for protein-based vaccine development in

25   which CTL responses are sought.

However, such "cross presentation" is generally thought to be
an inefficient process, and in many instances has led to
tolerance rather than immunity (reviewed in (1, 23). It has

30   been reported that the provision of T cell help can induce
immunity from a previously tolerogenically cross-presented
antigen (24, 25).

Another obstacle to the efficacy of help provided by T cells

35   in this manner is the scarcity of naïve T cells with
reactivity to unique antigens presented by the DC.

An alternative source of help is NKT cells, $\alpha\beta$ T cells with an
invariant TCR and intermediate level NK1 surface expression.
CD1d-restricted NKT cells expressing the invariant TCR α chain

5   encoded by Vα14Jα281 gene segments in mice, and Vα24JαQ in
humans, (NKT cells) are found in relative abundance in tissues
such as spleen, bone marrow, thymus and liver (12, 13).

Although the ligand for such invariant TCR cells has not been

10   defined, it is possible that NKT cells respond to a common
glycolipid presented by CD1d molecules that is induced as an
innate response to cellular distress. As NKT can express CD40L
and secrete significant levels of inflammatory cytokines, it
is possible that these cells can be readily induced to

15   activate DC (14). Recent studies have shown that human NKT
cell clones restricted to the group I CD1 molecules CD1a, CD1b
and CD1c, and $\gamma\delta$ T cells restricted by CD1c, can directly
induce maturation of cultured DC in vitro (16, 17).

20   Injection of purified DC has been used to obtain T-cell
response to antigen, but only in immunisation strategies using
short peptide antigens, not whole protein antigen (15). Using
whole antigen is preferable as it enables a range of peptides
to be presented, invokes both CD4+ and CD8+ T-cell responses

25   and may stimulate the secretion of protein specific
antibodies. In addition, the use of purified cells for
vaccination is a lengthy and complex procedure and not
practical for use in actual vaccination protocols. There is
thus a continuing need in the art for methods of improving T-

30   cell response to foreign antigen, particularly methods which
improve 'cross presentation' of antigen and thus lead to CD8+
T cell response as well as CD4+ cell response and antibody
secretion.

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Summary of the invention

The inventors have investigated whether NKT cells can influence immunological responses to soluble antigen in the presence of α-galactosyl ceramide (α-GalCer), a CD1d-binding glycolipid that has been shown to specifically induce activation of NKT cells (26). The inventors show that, while intravenous and oral administration of ovalbumin protein alone can induce activation of CD4$^+$ and CD8$^+$ T cells, these responses are considerably enhanced by concomitant activation of NKT cells leading to modulation of DC function.

The inventors have further shown that the enhanced immune response observed after stimulating NKT cells can be significantly further enhanced by stimulating simultaneously Toll Like Receptors (TLR) expressed on DC and B cells. TLR are pattern recognition receptors which bind bacterial products to alert the immune system of the presence of bacteria in the body.

Stimulation of NKT cells leads to the maturation of DC upon the interaction of CD40L (expressed by activated NKT cells) and CD40 (expressed by DC). The inventors have found that stimulation of TLR4 has a synergistic effect with the CD40 dependent stimulation pathway and results in a much stronger immune response. TLR4 can be stimulated by LPS and a derivative of LPS "MPL", which is non-toxic and can be injected into patients.

Injection of α-GalCer has been used to enhance anti-tumour immune responses, chiefly by activating NKT cells which kill tumour cells in an NK-like way (47) or by activating NK cells themselves (51). It has also been reported that the anti-tumour effect of α-GalCer may in part be due to stimulation of CD8+ T cells by IFNγ produced by activated NKT cells (50). WO03/009812 suggests the use of glycosylceramides, including

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α-GalCer, as activators of NKT cells in immunisation protocols
designed to elicit a T-cell response.

As described above, isolated and purified DC cells loaded with
antigenic peptide may be used for immunisation. In some
studies, peptide-expressing DC cells have been treated with α-
GalCer before reinjection (15).

However, this immunisation approach is of limited
applicability in practice. The multiple steps involved in
generation of DC (usually from bone marrow cells), culturing
the cells, loading with peptide and re-injecting into the host
are time-consuming and complex. In addition, using peptide
antigen means that only CD8+ or CD4+ T-cell responses are
invoked, depending on the MHC specificity of the peptide. It
also requires that the antigenic epitope of whatever antigen
it is desired to raise an immune response against be known.

The method of the present invention has several advantages.
Firstly, whole antigen is used for vaccination, so both CD4+,
CD8+ T-cell and antibody responses are obtained, resulting in
an enhanced T-cell response overall. Secondly, the strategy
is much more simple then existing strategies as it does not
require that DC cells be isolated, cultured or reinjected.
Thirdly, it requires preparation of only the soluble antigen -
the antigenic peptide need not be identified or purified.
Fourthly, it can be used with any individual, independent of
MHC haplotype.

Accordingly, at its most general, the invention provides
materials and methods for inducing an immune response to
soluble polypeptide antigen using an NKT cell activator.

In a first aspect of the present invention, there is provided
a composition for inducing an immune response in an individual

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to an antigen, said composition comprising said antigen in combination with an activator of NKT cells.

The invention also provides the use of an activator of NKT
5   cells in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual.

The invention also provides for a kit having first and second
10   containers, wherein the first container comprises a composition comprising an activator of NKT cells, and the second container comprises a composition comprising a soluble polypeptide antigen.

15   The activator of NKT cells may be a glycolipid presented by CD1d molecules or any molecule which activates NKT cells. Activation of NKT cells may be detected by, for example, increased expression of CD40L or secretion of cytokine such as IFNγ or IL-4.
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The activator of NKT cells may be a natural ligand of NKT cells. Alternatively, the activator of NKT cells may be α-GalCer, or an analogue of α-GalCer such as OCH (described in ref. 30) or alpha-glucosyl ceramide, or it may be a TCR
25   specific antibody. Methods for synthesis of α-GalCer are described by Figueroa-Perez and Schmidt, Carbohydrate Research 328 (2000) 95-102, and in US 6,555,372.

The immune response is preferably a T cell response. It may
30   be a CD4+ T cell response or CD8+ T cell response. Preferably, the immune response is both a CD4+ and a CD8+ T cell response. The immune response may also be an antibody response.

35   The antigen may be any antigen of choice, but is preferably a soluble protein.

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The composition may further comprise a TLR activator, that is
to say a substance which activates a Toll-like receptor. It
is believed that use of TLR activators in conjunction with
5   activators of NKT cells provide a synergistic adjuvant effect.
Preferably, the TLR activator is an activator of TLR3, TLR4,
TLR5, TLR7 or TLR9. A suitable TLR activator is MPL
(monophosphoryl lipid A), which binds TLR4. Other TLR
activators which may be used are LTA (lipoteichoic acid) and
10   PGN (peptidoglycan), which bind TLR2 and TLR6; Poly I:C
(polyinosine-polycytidylic acid), which binds TLR3; flagellin,
which binds TLR5; imiquimod (1-(2-methylpropyl)-1H-
imidazo(4,5-c)quinolin-4-amine), which binds TLR7 and CpG (DNA
CpG motifs), which binds TLR9; or any other component which
15   binds and activates a TLR. For more details, see Caetano Reis
e Sousa, Toll-like receptors and dendritic cells. Seminars in
Immunology 16:27, 2004.

However, use of TLRs in accordance with the invention is not
20   restricted to use in compositions comprising NKT cell
activators. The skilled person will appreciate that the NKT
cell activator and the TLR activator may be administered in
separate compositions.

25   Thus the invention provides the use of an activator of NKT
cells in the preparation of a medicament for inducing an
immune response to soluble polypeptide antigen in an
individual, wherein the medicament is for administration in
conjunction with a TLR activator.
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Also provided is the use of a TLR activator in the preparation
of a medicament for inducing an immune response to soluble
polypeptide antigen in an individual, wherein the medicament
is for administration in conjunction with an activator of NKT
35   cells.

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In these aspects of the invention, when the activator of NKT
cells is a glycosylceramide, the TLR activator is not CpG or
MPL.

5   The term glycosylceramide is used herein to refer to compounds
       of the formula I as disclosed in WO03/009812.

       Formula I:
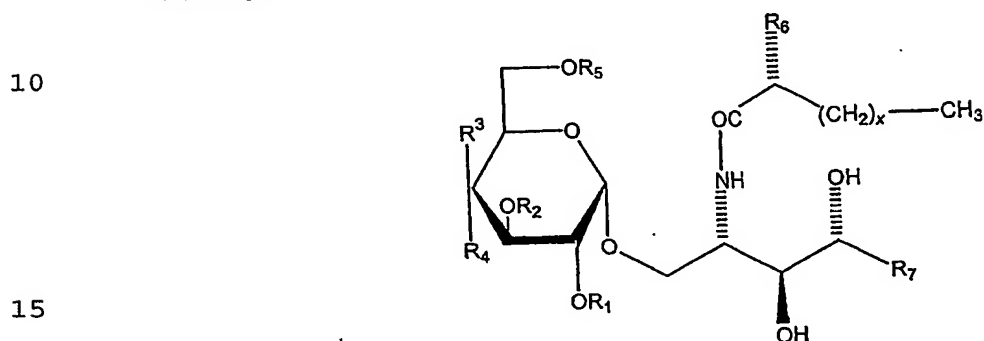
       wherein R_1, R_2 and R_5 represent H or a specific monosaccharide;
       R_3 and R_6 represent H or OH, respectively; R_4 represents H, OH
       or a specific monosaccharide; X denotes an integer from 1 to
20   23; R_7 represents any one of the following groups (a)-(g): (a) –
       $(CH_2)_{11}-CH_3$, (b) $-(CH_2)_{12}-CH_3$, (c) $-(CH_2)_{13}-CH_3$, (d) $-(CH_2)_9-$
       $CH(CH_3)_2$, (e) $-(CH_2)_{10}-CH(CH_3)_2$, (f) $-(CH_2)_{11}-CH(CH_3)_2$, (g) –
       $(CH_2)_{11}-CH(CH_3)-C_2H_5$.

25   The activator of NKT cells may be α-GalCer, OCH, α-
       glucosylceramide, or any of the other NKT activators referred
       to in this specification. αGalCer, α-glucosylceramide and OCH
       have the following formulae:

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α-GalCer

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α-glucosylceramide

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OCH

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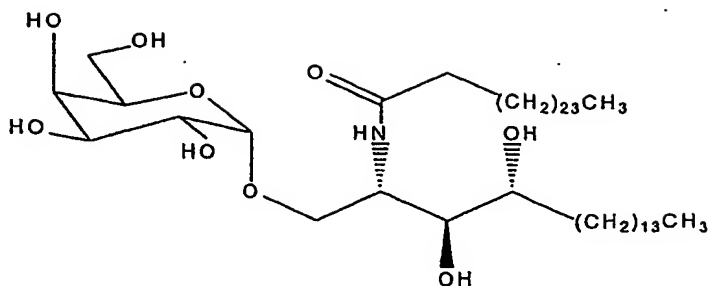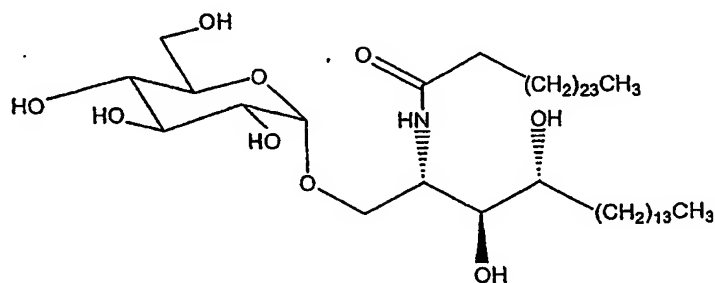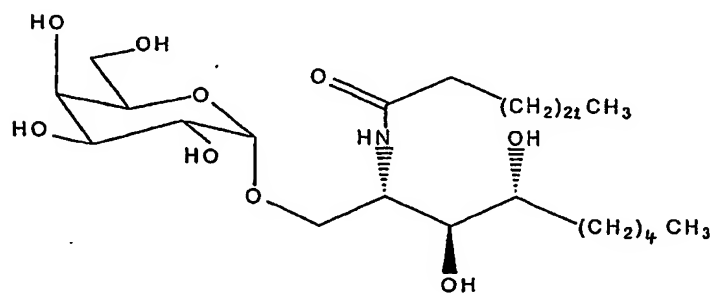
The TLR activator may be an activator of TLR3, TLR4, TLR5,
TLR7 or TLR9, including any of the specific activators
referred to above.

35   The invention further provides the use of α-GalCer in the
preparation of a medicament for inducing an immune response to

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The invention further provides the use of α-GalCer in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with CpG or
5   MPL.

The invention further provides the use of α-glucosylceramide in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual,
10   wherein the medicament is for administration in conjunction with CpG or MPL.

In a further aspect the invention provides a composition comprising an activator of NKT cells and a TLR activator, with
15   the proviso that, when the activator of NKT cells is a glycosylceramide, the TLR activator is not CpG or MPL.

Also provided is a composition comprising α-GalCer and MPL or CpG, and, further, a composition comprising α-glucosylceramide
20   and MPL or CpG.

The compositions of the invention may further comprise a purified soluble polypeptide antigen.

25   Also provided is a composition comprising OCH and a purified soluble polypeptide antigen.

In further aspects, the invention provides kits containing combinations of some or all of NKT cell activators, TLR
30   activators and soluble antigens.

Thus the invention provides a kit having first and second containers, wherein the first container comprises a composition comprising an activator of NKT cells, and the
35   second container comprises a composition comprising a TLR

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activator, with the proviso that, when the activator of NKT
cells is a glycosylceramide, the TLR activator is not CpG or
MPL.

5   Also provided is a kit having first and second containers,
       wherein the first container comprises a composition comprising
       α-GalCer, and the second container comprises a composition
       comprising CpG or MPL.

10   Also provided is a kit having first and second containers,
       wherein the first container comprises a composition comprising
       α-glucosylceramide, and the second container
       comprises a composition comprising CpG or MPL.

15   The kit may additionally have a third container, which
       container comprises a composition comprising a purified
       polypeptide antigen.

       Also provided is a kit having first and second containers,
20   wherein the first container comprises a composition comprising
       OCH, and the second container comprises a composition
       comprising a purified polypeptide antigen.

       In the kits of the invention, any or all of said compositions
25   may further comprise a pharmaceutically acceptable carrier or
       diluent.
       Thus, for example, the invention provides for a kit having
       first, second and third containers, wherein the first
       container comprises a composition comprising an activator of
30   NKT cells, the second container comprises a composition
       comprising a soluble polypeptide antigen, and the third
       container comprises a composition comprising a TLR activator.

       The compositions of the invention, whether or not provided as
35   part of kits, are preferably pharmaceutical compositions and,

if necessary, further comprise a pharmaceutically acceptable carrier or diluent. In one embodiment, the pharmaceutical composition may comprise a liposome which helps to target the peptide antigen to particular cells or tissue, e.g. lymphoid

5   tissue, and increases the half life of the antigen. Liposomes include emulsions, foams, micelles etc.

The pharmaceutical composition may also comprise at least one component which assists in the induction of any immune

10   response. The one or more components may include lipids, e.g. palmitic acid residues attached to the peptide antigen, or E.coli lipoproteins such as tripalmitoyl-S-glycerylcystein lyseryl-serine (P$_3$ CSS); and adjuvants, e.g. Freund's adjuvant.

15   The pharmaceutical composition according to the invention may be used as a vaccine. The vaccine may comprise one or more protein, polypeptide or peptide antigens in combination with the NKT cell activator. The one or more peptides may be linked to a carrier such as thyroglobulin, albumins such as

20   human serum albumin, tetanus toxoid, polyamino acids such as poly L-lysine, poly L-glutamic acid, influenza, hepatitis B virus care protein etc.

Alternatively, the peptide may be present in the vaccine as a

25   homopolymer comprising multiple copies of the same peptide, or as a heteropolymer of various peptides comprising antigens or epitopes of interest.

The vaccines may further comprise physiologically

30   tolerable/acceptable diluent such as water or saline, e.g. phosphate buffered saline. Further, as mentioned above the vaccine preferably includes an adjuvant such as incomplete Freund's adjuvant, aluminium phosphate, aluminium hydroxide, or alum.

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In a second aspect of the invention, there is provided a method of immunising an individual against an antigen, said method comprising the step of administering to said individual said antigen and a NKT cell activator, optionally in
5   combination with a TLR activator as already described.

The activator of NKT cells and the antigen may be administered simultaneously, preferably as a composition according the invention. Alternatively, the activator of NKT cells may be
10   administered before the polypeptide antigen is administered, preferably less than about 2 hours before. Likewise, the activator of NKT cells may be administered after the polypeptide antigen is administered, preferably less than about 8 hours after.
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The vaccine composition may be administered via injection, aerosol, oral, transdermal, transmucosal, intrapleural, intrathecal, or other suitable routes, e.g. intravenously, subcutaneously, intradermally, or intramuscularly.
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The present inventors have further determined that the enhanced immune response observed after stimulating NKT cells can be significantly further enhanced by stimulating simultaneously the TLR4, which is expressed on DC, and other
25   TLRs. Therefore, in all aspects of the invention, an activator of a TLR receptor may be used in combination with the activator of NKT cells. A suitable TLR activator is MPL, which binds TLR4. Other TLR activators which may be used are LTA and PGN, which bind TLR2 and TLR6; Poly I:C, which binds
30   TLR3; flagellin, which binds TLR5; imiquimod, which binds TLR7 and CpG, which binds TLR9; or any other component which binds and activates a TLR.

In one embodiment, the soluble antigen is a tumour antigen.
35   Alternatively, the antigen may be a polypeptide unique to a

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virus or bacterial cell, a parasite cell, or cellular
products.

Peptides that comprises the antigen or epitopes of interest
5   can be prepared synthetically, or by recombinant DNA
technology or from natural sources such as tumours or viruses.
Although the peptide will preferably be substantially free of
other naturally occurring host cell proteins and fragments
thereof, in some embodiments the peptides can be synthetically
10   conjugated to native fragments or particles.

Aspects and embodiments of the present invention will now be
illustrated, by way of example, with reference to the
accompanying figures. Further aspects and embodiments will be
15   apparent to those skilled in the art. All documents mentioned
in this text are incorporated herein by reference.

Description of the Figures

20   Figure 1. Intravenous injection of soluble antigen induces
functional T cell responses that are enhanced by co-
administration of α-GalCer.
Chicken ovalbumin suspended in PBS was injected into C57BL/6
mice (400 μg/mouse) together with either α-GalCer (1 μg/mouse)
25   or vehicle. (A) Induction of $CD8^+$ T cell responses specific for
the $OVA_{257-264}$ epitope was assessed in the blood by FACS analysis
using fluorescent tetrameric $H-2K^b/OVA_{257-264}$ complexes. FACS
profiles of naïve animals (control) and those receiving
ovalbumin $\pm$ α-GalCer or vehicle are shown. Mean proportions of
30   tetramer$^+$ cells as a percentage of $CD8^+$ cells ($\pm$ SEM) for each
treatment group are charted. (B) Thirteen days after ovalbumin
administration, cytolytic activity of the induced $OVA_{257-264}$-
specific response was assessed in vivo against various CFSE-
labeled syngeneic splenocytes loaded with titrated doses of
35      $OVA_{257-264}$-peptide as indicated, and a control splenocyte
population without peptide labeled with CMTMR. Representative

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FACS profiles for each treatment group are shown. Analysis of
antigen specific lysis was calculated at 16 h after target
cell administration, with percent specific lysis calculated as
the mean proportion of antigen loaded cells depleted relative
to control populations without antigen. (C) ELISpot assays
were used to assess induction of IFNγ by CD8$^+$ cells responding
to the MHC class I-binding peptide OVA$_{257-264}$, or CD4$^+$ T cells
responding to the class II-binding peptides OVA$_{265-290}$ and OVA$_{323-339}$, 11 days after ovalbumin administration. For these
analyses, splenocytes isolated from animals of the different
treatment groups were stimulated with each of the peptides in
vitro for 48 h.

**Figure 2.   Intravenous injection of soluble antigen ± α-GalCer
induces functional T cell responses with potent restimulatory
capacity.**
OVA$_{257-264}$-specific T cell responses in groups of animals (n = 5)
receiving intravenous injection with ovalbumin together with
either α-GalCer or vehicle, were restimulated with either **(A)**
i.v. injection of recombinant vaccinia encoding chicken
ovalbumin, or **(B)** i.v. injection of DC loaded with OVA$_{257-264}$-
peptide. Specific responses were assessed in the blood by FACS
analysis using fluorescent tetrameric H-2K^b/OVA$_{257-264}$ complexes
seven days after restimulation. Charts showing mean responses
for each treatment group (± SEM) prior to restimulation (left
panels) and after restimulation (right panels) are presented.
Control groups without prior ovalbumin treatment served to
control for efficacy of the secondary stimulus, and naïve
animals, or animals treated with DC without peptide, served as
negative controls.

**Figure 3.   Enhancement of T cell responses to soluble antigen
with injection of α-GalCer requires the involvement of NKT
cells.**
OVA$_{257-264}$-specific T cell responses were assessed in the blood
using H-2K^b/OVA$_{257-264}$ tetramers in groups (n = 5) of wildtype

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C57BL/6 animals and **(A)** NKT$^{-/-}$ mice, or **(B)** CD1d$^{-/-}$ mice, seven days after receiving intravenous injection with ovalbumin together with either vehicle or α-GalCer.   **(C)** OVA$_{257-264}$-specific T cell responses were likewise assessed in wildtype animals receiving vehicle, α-GalCer or β-GalCer, a glycolipid known to bind CD1d but unable to stimulate NKT cells (36).

Figure 4. Injection of α-GalCer enhances T cell responses to soluble protein antigen only if administered in close temporal association with protein.
OVA$_{257-264}$-specific T cell responses were assessed in the blood seven days after groups of animals (n = 5) received intravenous injection with ovalbumin (time zero = 0 h) and α-GalCer or vehicle injected at the indicated times relative to protein injection.

Figure 5. DC-induced T cell responses can be enhanced by concomitant, α-GalCer-mediated, activation of NKT cells capable of substituting for CD4$^+$ T cell help.
(A) LCMV GP$_{34-41}$-specific CD8$^+$ T cell responses were assessed in the blood of groups of animals (n =5) seven days after they had received DC loaded with LCMV GP$_{34-41}$ together with α-GalCer, vehicle or PBS, or untreated DC. One group (first panel) received DC that were loaded with LCMV GP$_{34-41}$ and α-GalCer separately, and then combined before injection. In some instances, NKT$^{-/-}$ mice were used as recipients (right panel), and in others the DC were matured with LPS prior to injection (lower panel). **(B)** OVA$_{257-264}$-specific T cell responses were assessed in animals that received α-GalCer- or vehicle-treated DC loaded with the MHC class I-binding peptide OVA$_{257-264}$ alone, or together with the class II-binding peptide OVA$_{323-339}$. Animals injected with DC without peptides served as negative controls.
(C) OVA$_{257-264}$-specific T cell responses were assessed in animals that received α-GalCer- or vehicle-treated DC exposed to whole ovalbumin for 20 h. Animals injected with DC without protein served as negative controls.

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Figure 6. Injection of α-GalCer leads to maturation of splenic dendritic cells and increases cross presentation. Antibody conjugated to magnetic beads was used to isolate CD11c⁺ cells from the spleens of animals that had received i.v injection of ovalbumin together with vehicle, α-GalCer or LPS 24 h previously. Representative FACS profiles showing expression of CD80 and CD86 on positively sorted CD11c⁺ cells from ovalbumin ± α-GalCer treated animals relative to levels on CD11c⁺ cells from naïve controls (grey).

Figure 7. Activation of NKT cells enhances T cell responses to soluble protein via a CD40-mediated mechanism independent of IFNγ production.
OVA$_{257-264}$-specific T cell responses were assessed in the blood seven days after groups (n = 5) of C57BL/6 or CD40L$^{-/-}$ mice received intravenous injection with ovalbumin together with either vehicle or α-GalCer (A), with serum cytokine levels in α-GalCer-treated animals determined by ELISA at the indicated times (B). OVA$_{257-264}$-specific T cell responses were similarly assessed in IFNγR$^{-/-}$ animals and 129S6/SvEv controls (C), or in C57BL/6 animals injected with ovalbumin and the α-GalCer analogue OCH (D). Serum cytokine levels were determined for the latter experiment (E).

Figure 8. Oral administration of soluble antigen and alpha-GalCer induces functional CTL. (a) Whole OVA was administered by gavage (30 mg/mouse) together with either alpha-GalCer (8 μg/mouse) or vehicle. Induction of OVA$_{257-264}$-CD8⁺ T cell responses was assessed in the blood 7 days later. (b) Cytolytic activity of the induced OVA$_{257-264}$-specific response was assessed in vivo 11 days after OVA administration against various CFSE-labeled syngeneic splenocytes loaded with titrated doses of OVA$_{257-264}$-peptide as indicated, and a control splenocyte population without peptide labelled with CMTMR.

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Representative FACS profiles for each treatment group are
shown. Analysis of antigen specific lysis was calculated at 16
h after target cell administration, with percent specific
lysis calculated as the mean proportion of antigen loaded
5   cells depleted relative to control populations without
antigen.

Figure 9. Co-stimulation of TLR4 on DC further enhances T-cell
response to soluble protein
10   Liposomes containing alphaGalcer + ovalblumin + MPL (an LPS
derivative which binds TLR4) were injected i.v. into mice and
7 days later frequency of OVA specific CTL was measured by ex-
vivo tetramer staining. Liposomes containing either ovalbumin
and MPL or ovalbumin and alphaGalceramide were used as
15   control. FACS profiles of naïve animals and those receiving
ovalbumin +/- α-GalCer and/or MPL are shown. Mice immunized
with liposomes containing OVA alone showed: 0.1% of tetramer+
cells; OVA + alphaGalcer: 2% of tetramer+ cells;
OVA+alphaGalcer+MPL: 13% of tetramer+ cells.

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Figure 10. Synergistic effect of several TLR ligands in
combination with alphaGalactosylceramide in inducing mature
DC.

Injection of iNKT cell ligands leads to maturation of splenic
25   DCs, increasing their immunostimulatory capacity. Animals
were treated i.v. with either 100 ng of iNKT cell ligand
alpha-GalCer, or 25 μg of TLR4 ligand monophosphoryl lipid A
(MPL), or 2.5 μg.poly I:C, or 2.5 μg.flagellin, or 50 μg.CpG.
Surface expression of CD86 was assessed on splenic CD11c$^+$ cells
30   24 h later.

Figure 11. Activation of NKT cells enhances T cell responses
to tumour-specific antigen.

Progression of EG.7OVA tumours implanted subcutaneously were
monitored in animals that were subjected to; (i) no treatment,
(ii) 400 μg ovalbumin together with vehicle 8 days prior to
challenge (iii) 400 μg ovalbumin together with 1 μg α-GalCer 8
5   days prior to challenge. Mean tumour sizes per group (n = 5) ±
SEM are shown.

**Figure 12. Injection of αOCH with MPL leads to further
maturation of splenic dendritic cells and increases expansion
10   of cytolytic OVA specific CTL.** Antibody conjugated to magnetic
beads was used to isolate CD11c$^+$ cells from the spleens of
animals that had received i.v injection of ovalbumin together
with vehicle, OCH or OCH with MPL 24 h previously.
Representative FACS profiles showing expression of CD86 on
15   positively sorted CD11c$^+$ cells are shown.

**Figure 13. Co-stimulation of TLR4 on DC further enhances T-
cell response to soluble protein and OCH**
OCH + ovalblumin + MPL were injected i.v. into mice and 7 days
20   later frequency of OVA specific CTL was measured by ex-vivo
tetramer staining. A) FACS profiles of naïve animals and
those receiving ovalbumin +/- OCH and/or MPL are shown. B)
Mean proportions of tetramer$^+$ cells as a percentage of CD8$^+$
cells (± SEM) for each treatment group are charted.
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**Figure 14. Injection of OCH and MPL leads to maturation of
splenic DCs, increasing their immunostimulatory capacity.**
Animals were treated i.v. with either 1 μg of OCH, or 25 μg of
MPL, or OCH plus MPL. The immunostimulatory capacity of CD11c$^+$
30   cells isolated from the spleens of these group of animals was
assessed by loading with influenza NP $_{366-374}$ peptide ex vivo,
and then by adding splenocytes transgenic for the NP $_{366-374}$
specific T cell receptor. A) proliferation of CFSE labelled
transgenic splenocytes is shown B) secretion of gamma
35   interferon by transgenic splenocytes is shown.

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Detailed Description of the Invention

Pharmaceutical Compositions

The NKT activators of the invention may be formulated in
pharmaceutical compositions, and administered to patients in a
variety of forms.

Pharmaceutical compositions for oral administration may be in
tablet, capsule, powder or liquid form. A tablet may include
a solid carrier such as gelatin or an adjuvant or an inert
diluent. Liquid pharmaceutical compositions generally include
a liquid carrier such as water, petroleum, animal or vegetable
oils, mineral oil or synthetic oil. Physiological saline
solution, or glycols such as ethylene glycol, propylene glycol
or polyethylene glycol may be included. Such compositions and
preparations generally contain at least 0.1wt% of the
compound.

Parenteral administration includes administration by the
following routes: intravenous, cutaneous or subcutaneous,
nasal, intramuscular, intraocular, transepithelial,
intraperitoneal and topical (including dermal, ocular, rectal,
nasal, inhalation and aerosol), and rectal systemic routes.
For intravenous, cutaneous or subcutaneous injection, or
injection at the site of affliction, the active ingredient
will be in the form of a parenterally acceptable aqueous
solution which is pyrogen-free and has suitable pH,
isotonicity and stability. Those of relevant skill in the art
are well able to prepare suitable solutions using, for
example, solutions of the compounds or a derivative thereof,
e.g. in physiological saline, a dispersion prepared with
glycerol, liquid polyethylene glycol or oils.

In addition to one or more of the compounds, optionally in
combination with other active ingredient, the compositions can
comprise one or more of a pharmaceutically acceptable

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excipient, carrier, buffer, stabiliser, isotonicizing agent,
preservative or anti-oxidant or other materials well known to
those skilled in the art. Such materials should be non-toxic
and should not interfere with the efficacy of the active
5   ingredient. The precise nature of the carrier or other
material may depend on the route of administration, e.g.
orally or parenterally.

Liquid pharmaceutical compositions are typically formulated to
10   have a pH between about 3.0 and 9.0, more preferably between
about 4.5 and 8.5 and still more preferably between about 5.0
and 8.0. The pH of a composition can be maintained by the use
of a buffer such as acetate, citrate, phosphate, succinate,
Tris or histidine, typically employed in the range from about
15   1 mM to 50 mM. The pH of compositions can otherwise be
adjusted by using physiologically acceptable acids or bases.

Preservatives are generally included in pharmaceutical
compositions to retard microbial growth, extending the shelf
20   life of the compositions and allowing multiple use packaging.
Examples of preservatives include phenol, meta-cresol, benzyl
alcohol, para-hydroxybenzoic acid and its esters, methyl
paraben, propyl paraben, benzalconium chloride and
benzethonium chloride. Preservatives are typically employed
25   in the range of about 0.1 to 1.0 % (w/v).

Experimental Procedures

Mice
30   C57BL/6, 129S6/SvEv and CD40L$^{-/-}$ mice were from breeding pairs
originally obtained from Jackson Laboratories, Bar Harbor,
Maine. Also used were mice lacking the Jα281 TCR gene segment
(27) which were devoid of Vα14$^+$ NKT cells while having other
lymphoid cell lineages intact (referred to in the text as NKT$^-$
35     $^/$ mice), CD1d$^{-/-}$ mice (28) and IFN$\square$R$^{-/-}$ mice (29). All animals
were maintained at the Biomedical Services Unit of John

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Radcliffe Hospital by brother x sister mating; in vivo
experimental protocols were performed according to
institutional guidelines.

In vitro culture media and reagents

5   Unless otherwise stated all cultures were maintained in
complete medium (CM) comprising of RPMI (Sigma-Aldrich,
Dorset, England) supplemented with 2 mM glutamine, 1 %
penicillin-streptomycin, 5×10^{-5} M 2-mercapto-ethanol (all
10   Invitrogen Ltd, Paisley, UK) and 10 % fetal bovine serum
(Globepharm, Guildford, England). Chicken ovalbumin grade VII
was from Sigma-Aldrich. Peptides derived from chicken
ovalbumin, including $OVA_{257-264}$ (binding to $H-2K^b$), $OVA_{323-339}$ and
$OVA_{265-280}$ (both binding and $I-A^b$), and peptide $LCMV_{34-41}$ ($H-2K^b$)
15   derived from lymphocytic choriomeningitis virus glycoprotein,
were prepared in-house. The glycolipids α-galactosylceramide
(α-GalCer) and β-galactosylceramide (β-GalCer) were from Kirin
Brewery Co. Ltd, Japan and Fluka Chemie AG, Switzerland
respectively. The glycolipids were solubilized in 0.5 %
20   Tween/PBS, hereafter referred to as 'vehicle'.

Administration of soluble antigen

Ovalbumin protein was diluted in PBS and admixed with PBS-
diluted glycolipid solutions (α-GalCer, β-GalCer or OCH), or
25   PBS-diluted vehicle, immediately prior to injection into the
lateral vein. Each animal received 400 μg protein and either 1
μg of glycolipid, or equivalent final volume of vehicle.

Immunization with antigen-loaded DC

30   Bone marrow cells from C57BL/6 mice were cultured in 20 ng/ml
IL-4 and 20 ng/ml GM-CSF for 7 d. Cultures typically contained
70-100 % DC as determined by fluorescent staining with the
anti-CD11c (BD Pharmingen, San Diego, CA). DC were loaded with
peptide by incubation in CM containing 10 μM synthetic peptide
35   for 2 h, or loaded with whole protein by incubation in CM
containing 100 μg/ml protein for 24 h. DC were loaded with α-

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GalCer by incubation in CM containing 100 ng/ml for 40 h.
Cells were washed to remove excess Ag prior to i.v. injection
(6×10^5 DC per animal).

5   **Infection with recombinant vaccinia virus**
        Mice were infected with 10^6 plaque forming units of the
        recombinant virus VV-OVA (31), expressing full length chicken
        ovalbumin, by intravenous injection in PBS.

10   **Monitoring CTL responses with MHC tetramers**
        Tetrameric H-2 K^b/OVA$_{257-264}$ peptide, or tetrameric H-2 K^b/LCMV$_{34-41}$
        peptide complexes were prepared as outlined in Altman et al.
        (32), and used to stain fresh PBL isolated from the lateral
        tail vein. Approximately 5×10^5 PBL were suspended in 20 μl CM
15   and incubated with 0.5 μg of tetramer complexes at 37 °C for
        20 min. The cells were then incubated with anti-CD8α (BD
        Pharmingen) for 10 min at 4 °C, washed twice with PBS, and
        resuspended in PBS for FACS analysis. Cells were analysed with
        FACScan hardware and CellQuest software (BD Biosciences ,
20   Mountain View, CA).

        ELISpot
         Single cell suspensions were prepared from murine spleens by
        gently teasing tissue through nylon gauze with a syringe
25   plunger. The cell suspensions were treated with RBC lysis
        solution (Puregene, Gentra Systems, Minneapolis, USA) to
        deplete red blood cells, and resuspended in CM. Analysis of
        murine IFNγ-production by graded doses of splenocytes in
        response to stimulation with 10 μM peptide for 48 h was
30   performed on MultiScreen-IP high protein binding 96-well
        plates (Millipore Corporation, Bedford, MA, USA) using MabTech
        mouse IFNγ ELISpot kit according to the manufacturers
        instructions. For all ELISpot analyses, splenocytes from each
        sample were stimulated with 20 μg/ml Concanavalin A to serve
35   as positive controls for ability to produce IFNγ.

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In vivo cytotoxicity assay

Cytotoxicity was assessed on fluorescence-labeled syngeneic
spleen cell populations loaded with specific antigen
administered by i.v. injection. Groups of immunized mice (n =
5) , and a non-immunized control group (n = 5) were injected
with a mixture of four differentially labeled populations; a
control population without antigen fluorescence-labeled with
the dye CMTMR (10 μM), and three target populations labeled
with CFSE at different concentrations (1.65 nM, 0.3 nM and
0.07 nM), and loaded with titrated doses of specific peptide
(5 μM, 500 nM, 50 nM). Cytotoxicity was assessed by FACS
analysis on blood taken from the lateral tail vein. The mean
percent survival of peptide-loaded targets cells was
calculated relative to antigen-negative controls. Cytotoxic
activity was then expressed as percent specific lysis,
calculated by the equation 100 - mean percent survival of
peptide-loaded targets.

Phenotypic analysis of splenic DC

DC were isolated from the spleens of animals that had been
injected with α-GalCer or vehicle by using anti-CD11c magnetic
beads (Miltenyi Biotech, Gladbach,Germany) according to the
manufacturer's instructions. The isolated cells were assessed
for expression of CD8$\square$, CD8$^-$ and CD86 with specific monoclonal
antibodies (BD Pharmingen).

ELISA. Serum cytokine levels were determined by ELISA using
commercial ELISA kits (R&D Systems, Minneapolis, USA)
following the manufacturers instructions.

Tumour immunity assay

Eight days after administration of ovalbumin $\pm$ α-GalCer or
vehicle, groups of mice (n = 5) were challenged with EG7.OVA
cells expressing a cDNA encoding the chicken ovalbumin
sequence (33). Challenge was with 1 x 10^6 tumour cells injected
s.c. into the flank. Mice were monitored for tumour growth
every 3-4 days, and tumour size for each group was calculated

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as the mean of the products of bisecting diameters (± SEM).
Measurements were terminated for each group when the first
animal developed a tumour in excess of 200 mm^2.

5   Results

       Administration of α-GalCer together with soluble antigen
       provides potent immune responses.
       The possibility that NKT cells have the potential to influence
10   the quality of adaptive immune responses was investigated in
       the context of T cell priming via intravenous delivery of
       soluble antigen. Immune responses were therefore monitored in
       C57BL/6 mice injected with chicken ovalbumin protein in the
       presence or absence of the NKT cell ligand α-GalCer. This
15   glycolipid has been shown to rapidly induce the release of a
       NKT-cell derived cytokines, leading to a 'cytokine storm'
       characterized by high levels of IFNγ and IL-4 in the serum
       (26, 34). In agreement with these studies, co-administration
       of 1 μg of α-GalCer with 400 μg ovalbumin induced a
20   substantial increase of IFNγ in the serum over a 48 h period.
       Of particular interest was the possibility that NKT cell
       mediated signals, be they soluble factors or cognate
       interactions, could lead to cross presentation of peptides
       derived from the administered ovalbumin protein to peptide-
25   specific CD8$^+$ T cells, thereby perhaps providing a potent,
       specific, CTL response. For this reason, immune responses were
       initially screened in the blood using MHC class I/peptide
       tetramers capable of highlighting CD8$^+$ T cells reactive to the
       H-2 K^b-binding peptide OVA$_{257-264}$. The result of these analyses,
30   presented in Figure 1A, indicated that co-administration of
       ovalbumin and α-GalCer led to a significant increase in OVA$_{257-264}$-specific CD8$^+$ T cells in the blood over animals given
       ovalbumin and vehicle (a polysorbate solution in which the
       glycolipid was solubilized). Repeated experiments indicated
35   that co-administration of α-GalCer with ovalbumin led to
       between 5- and 10-fold increases in OVA$_{257-264}$-specific CD8$^+$ T

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cells in the blood. Thus, while cross presentation of peptides
derived from ovalbumin was observed in the absence of
activation of NKT cells, the levels of cross presentation were
markedly increased by concomitant stimulation of NKT cell

5   activity.

The functional state of the induced ovalbumin-reactive CD8$^+$ T
cells was investigated by assessing cytotoxic capacity,
cytokine induction and ability to respond to further antigenic

10   stimulation. Cytotoxic capacity was assessed against OVA$_{257-264}$
peptide-loaded syngeneic splenocyte targets that had been
labeled with fluorescent dye and injected into the lateral
vein 13 days after initial priming with ovalbumin ± α-GalCer.
Specific lysis of the antigen-loaded population was monitored

15   relative to a differentially labeled control splenocyte
population that was not loaded with peptide. This assay,
presented in Figure 1B, indicated that the ovalbumin-specific
CD8$^+$ T cells induced were indeed capable of mediating specific
cell lysis. In fact, cytotoxic activity was observed in all

20   ovalbumin treated animals regardless of whether α-GalCer was
administered or not. However, where α-GalCer was used, the
cytotoxic response was markedly greater, a likely reflection
of the overall increase in the specific CTL pool in these
animals.

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As a further assessment of functional activity, peptide-
specifc ELISPOT assays were used to assess IFNγ production
from the induced OVA$_{257-264}$ CD8$^.$ T cell population. In addition,
ELISPOT assays were also used to determine whether responses

30   to the MHC class II binding peptides OVA$_{323-339}$ and OVA$_{265-280}$ were
similarly affected by α-GalCer treatment. For these analyses
splenocytes were taken from animals 11 days after antigen
administration and stimulated with the specific peptides in
vitro for 48 h (Fig. 1C). IFNγ responses to the class I

35   peptide, OVA$_{257-264}$, were in agreement with the MHC class I
tetramer data, with co-administration of α-GalCer and

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ovalbumin leading to increased numbers of specific IFNγ-
producing cells over levels found in animals treated with
ovalbumin and vehicle (3-fold increase). This same trend was
observed for both of the MHC class II binding peptides tested.
5   In fact, very similar numbers of IFNγ-producing cells were
seen for each of the treatment groups, regardless of peptide
used. Thus, CD4$^+$ T cell responses are also increased by α-
GalCer treatment.

10   As a final assessment of functional activity, the ability of
the induced ovalbumin-specific T cells to respond to further
stimulation in vivo was examined. Animals that had been
treated with ovalbumin ± α-GalCer, or naïve controls, were
therefore challenged with either OVA$_{257-264}$-loaded DC, or
15   vaccinia encoding the whole chicken ovalbumin DNA sequence
(Fig. 2). In both instances, OVA$_{257-264}$-specific populations were
greatly expanded one week after restimulation in vivo. The
responses were proportional to the initial responses following
protein administration, so that in both assays the groups
20   initially treated with ovalbumin and α-GalCer produced the
most significant restimulatory responses. OVA$_{257-264}$-specific
CD8$^+$ T cells induced in the absence of α-GalCer could also be
restimulated, but the smaller populations induced likely
reflect the initially smaller populations induced in the
25   absence of α-GalCer.

These results show that administration of α-GalCer greatly
enhanced take-up and presentation of blood borne ovalbumin
protein to CD4$^+$ cells and to CD8$^+$ T cells.
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**NKT cells are required for the enhancing effect of α-GalCer on
T cell responses induced with soluble antigen**
While it has been clearly established that α-GalCer is a CD1d-
binding ligand of NKT cells, it was still possible that the
35   increased immune responses to i.v. injection of ovalbumin
protein seen in the presence of α-GalCer was not directly

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related to stimulation of activity from CD1d/α-GalCer-reactive
NKT cells. For example, it was possible that increased T cell
responses may result from interaction between ovalbumin
protein and the glycolipid leading to efficient absorption of
5   antigen by resident APC, perhaps via CD1d molecules.
Therefore, to provide further proof that NKT cells were
required for the α-GalCer-mediated increase in presentation to
T cells (including cross presentation to CD8$^+$ T cells), immune
responses to ovalbumin in animals that lack the Jα281 gene
10   segment and consequently do not harbor NKT cells that can
react to α-GalCer (27) were examined. As shown in Fig. 3A, co-
administration of ovalbumin and α-GalCer to these NKT$^{-/-}$
animals did not lead to the increased activities seen in the
wild-type animals as assessed by monitoring levels of OVA$_{257-264}$
15   CD8$^+$ T cells responses with tetramer in the blood on day 7 post
ovalbumin injection, and by IFNγ ELISPOT assays on splenocytes
taken on day 10. As a further assessment of the involvement of
NKT cell activation in the enhancement of T cell responses to
blood-borne protein, ovalbumin was injected into CD1d-
20   deficient mice unable to present the α-GalCer glycolipid to
NKT cells. Again, co-administration α-GalCer into CD1d$^{-/-}$
animals was not able to enhance ovalbumin-induced T cell
responses (Fig. 3B). Finally, to examine the possibility that
the glycolipid itself was directly enhancing antigen uptake of
25   ovalbumin protein, ovalbumin was injected into wild-type
animals together with α-GalCer, a glycolipid with similar
structure to α-GalCer shown to bind to CD1d molecules, but
without capacity to induce NKT cell activation (36). Co-
administration of α-GalCer and protein did not lead to the
30   dramatically enhanced levels of specific T cell responses that
had been observed with α-GalCer. This shows that the enhanced
responses observed with α-GalCer can be directly attributed to
the activation of NKT cells.

35   **The enhancing effect of NKT stimulation is dependent on timing
of administration of α-GalCer relative to protein**

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The kinetics of NKT activation, cytokine release and
subsequent activation induced cell death following α-GalCer
injection have been well documented (26, 35, 37). Screens were
performed to establish at which time-point(s) within these
5   kinetics that the most significant enhancing effect of α-
GalCer on immune responses to soluble antigen was observed. In
the first screen, hallmark time-points for injection of α-
GalCer relative to ovalbumin were chosen on the basis of the
known serum cytokine profiles in response to α-GalCer. Thus,
10   ovalbumin was injected 2 h after α-GalCer injection when IL-4
levels were known to be peaking in the serum, and 24 h after
α-GalCer injection, when IFNγ levels were peaking. In both of
these situations, α-GalCer administration had no enhancing
effect on $OVA_{257-264}$ $CD8^+$ T cells responses measured by tetramer
15   in the blood on day 7 post-ovalbumin injection (Fig. 4A). In
addition, α-GalCer injected 24 h after ovalbumin did not
enhance the ovalbumin-specific T cell responses. In fact,
enhanced responses were only observed when the ovalbumin and
α-GalCer were injected at the same time (Fig. 4A). However, a
20   finer analysis of α-GalCer administration, between 2 h before
and 8 h after ovalbumin administration, indicated that some
enhancement could be observed if α-GalCer was administered
within this period, with this effect diminishing 8 h after α-
GalCer administration (Fig. 4B). Again, the most potent
25   enhancement was observed when the ovalbumin and α-GalCer were
injected at the same time. These data imply that enhancement
of T cell responses may be dependent upon close temporal
association between presentation of ovalbumin-derived peptides
and α-GalCer molecules.

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**The enhancing effect of NKT cell stimunlation is dependent
upon simultaneous presentation of α-GalCer and processed
protein antigen by dendritic cells.**
That NKT induced enhancement of T cell responses required
35   close timing of α-GalCer and protein administration could
reflect the need for simultaneous uptake of these molecules,

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and perhaps a requirement for co-localization of their
antigenic components on the same APC. In order to look at this
possibility in more detail, immune responses to injection of
cultured bone marrow-derived DC loaded with glycolipid and
5   peptide antigens in vitro were examined. Intravenous injection
of DC loaded with α-GalCer has been reported to induce
cytokine release in vivo in much the same way as injection of
free α-GalCer, although with some changes to the kinetics of
release (38). Loading DC with α-GalCer in vitro did not induce
10   maturation prior to injection, as determined by expression
levels of CD80, CD86 and MHC class II (data not shown). Immune
responses to the MHC class I-binding peptides LCMV GP$_{34-41}$ (from
lymphocytic choriomeningitis virus glycoprotein) (Fig. 5A) or
OVA$_{257-264}$ (Fig. 5B) were assessed in the blood with MHC
15   tetramers seven days after DC injection. For both of the MHC
class I-binding peptides used, measurable immune responses
were detected in animals injected with peptide loaded DC, as
was expected. Importantly, in both experimental models,
loading DC with peptide and α-GalCer resulted in reproducible
20   2-3 fold increases in immune responses (Fig. 5A + B). When
immune responses to LCMV GP$_{34-41}$ peptide were assessed in more
detail, it was found that the α-GalCer-induced increase in
immune response occurred only if the peptide and α-GalCer were
presented on the same DC, rather than on separate DC (Fig. 5A,
25   first panel). In addition, the enhancing effect of
simultaneous presentation of peptide and α-GalCer was not seen
when the DC were injected into NKT$^{-/-}$ hosts (Fig 5A, right
panel), implying a direct role for the interaction of NKT with
DC in producing this effect, presumably resulting in DC
30   maturation. By maturing DC with LPS prior to injection, it was
possible to enhance the immune response to LCMV GP$_{34-41}$ peptide
without addition of α-GalCer, with the level of enhancement
similar to that seen with non-matured DC loaded with α-GalCer.
However, when α-GalCer was loaded onto DC that had already
35   been matured with LPS, no further increase in immune response
was observed to LCMV GP$_{34-41}$. Together these data suggest that

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the effect of loading DC with peptide and α-GalCer was to
permit an interaction between DC and NKT cell that leads to DC
maturation, and hence an improvement of the MHC class I-
restricted immune response. This observation is very similar

5   to the reported function of CD4$^+$ T cell help in the context of
CD8$^+$ T cell activation (8-10). While the DC used in our
experiments were cultured in FCS, and are therefore likely to
be presenting some unique peptides to CD4$^+$ T cells in vivo, it
would appear that this CD4$^+$ T cell help is insufficient to

10   provide optimum DC maturation. We therefore examined whether
addition of a known MHC class II-binding peptide would provide
a more potent CD4-mediated maturation stimulus to DC, and
whether α-GalCer-mediated NKT stimulation can fully substitute
for this CD4 T cell help. The results, presented in Figure 5B,

15   indicate that injection of DC loaded with both MHC class I
binding peptide (OVA$_{257-264}$) and MHC class II binding peptide
(OVA$_{323-339}$) resulted in a larger OVA$_{257-264}$-specific immune
response than DC loaded with the class I binding peptide
alone. However, this CD4$^+$ T cell-mediated help was not as

20   significant as the response observed if the class II binding
peptide is substituted with the addition of α-GalCer. Indeed,
the α-GalCer-mediated enhanced response could not be improved
even if α-GalCer was used together with both class I- and
class II- binding peptides. Thus, it would appear that NKT are

25   able to exert a 'helper' function that exceeds the potency of
CD4$^+$ T cell mediated help. Finally, the effect of α-GalCer-
mediated NKT activity was examined in the context of
presentation of whole protein. DC were exposed to ovalbumin
protein for 24 h before injection, a process that permitted

30   some degree of antigen uptake and processing. A weak OVA$_{257-264}$-
specific, response was observed in the blood seven days after
DC injection, although the levels seen were statistically
insignificant (Fig. 5C). However, if the ovalbumin-loaded DC
were also loaded with α-GalCer, a significant immune response

35   was observed. Loading with ovalbumin was done in the absence
of NKT cells, so this result could not be attributed to an α-

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GalCer-mediated NKT function that affects antigen uptake. It
is therefore most likely that increased immune response
reflects NKT cell-mediated maturation of DC in vivo. Again, as
antigen processing should have provided both MHC class I- and
5   class II- binding peptides in this experiment, the effect of
NKT cell-mediated 'help' appears to be more potent than
classical CD4$^+$ T cell-mediated help.

Injection of α-GalCer leads to maturation of splenic dendritic
10   **cells and increased cross presentation**
To further investigate a role for NKT cell-mediated maturation
of DC in vivo, the maturation status of DC taken directly from
the spleens of animals injected with ovalbumin ± α-GalCer was
examined. DC were isolated from the spleens of animals from
15   both groups by positive selection using anti-CD11c coated
magnetic beads 24 h after antigen injection. As a positive
control on induction of DC maturation in vivo, a third group
of animals was injected with lipopolysaccharide (LPS), a
stimulus that has been shown to induce upregulation of co-
20   stimulatory molecules on splenic DC in vivo (39). Maturation
status was established by determining levels of expression of
CD80 and CD86 on the isolated DC. Representative FACS profiles
are shown in Fig. 6A. While injection of vehicle had no effect
upon levels of CD80 and CD86 on DC relative to naïve controls,
25   injection of α-GalCer induced an upregulation of expression of
these co-stimulatory molecules that was equal to LPS, or in
the case of CD80, even greater than LPS. These data suggest,
therefore, that NKT cell activation provides a potent DC
maturation stimulus.
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The enhancing effect of NKT cell stimulation is CD40L
dependent, but does not require INFγ
It has previously been reported that the provision of help by
CD4$^+$ T cells is through interaction of T helper cells with DC,
35   via CD40. CD40L is upregulated on CD4$^+$ T cells in response to T
cell activation, and signals mediated by via CD40 on DC lead

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to DC activation. NKT cells have also been reported to express
CD40L upon activation (40) and it has therefore been suggested
that NKT cells may also induce DC maturation and activation
through interaction with CD40 (14, 16). To investigate the
5   possibility that the enhanced ovalbumin-specific T cells
responses induced with α-GalCer are CD40-mediated, ovalbumin
protein was injected into CD40L$^{-/-}$ recipients together with α-
GalCer or vehicle. OVA$_{257-264}$-specific CD8$^+$ T cell responses were
monitored in the blood seven days later with tetramers. The α-
10   GalCer-mediated enhancement of responses to ovalbumin
administration was not observed in CD40L$^{-/-}$ recipients (Fig.
7A). Analysis of cytokine levels in serum in these recipients
indicated that whereas α-GalCer induced both IFNγ and IL-4
release in wild-type animals, only IL-4 was induced in CD40L$^{-/-}$
15   recipients (Fig. 7B). Thus, while NKT cells were activated in
CD40L$^{-/-}$ animals, they were unable to produce of IFNγ and
enhance T cell responses in the absence of subsequent
CD40/CD40L mediated signals.

In order to assess the possibility that IFNγ played a key
20   role in NKT modulation of T cell responses, ovalbumin protein
was injected into IFNγR$^{-/-}$ recipients together with α-GalCer or
vehicle (Fig 7C). Lack of a receptor for IFNγ made no
difference to the induction of OVA$_{257-264}$-specific CD8$^+$ T cell
responses measured in the blood, and importantly, did not
25   affect the enhancement of this response by α-GalCer.
Similarly, injection of ovalbumin protein together with OCH
(Fig. 7D), an analogue of α-GalCer that induces activation of
NKT cells with a profile of cytokine release predominated by
IL-4 rather than IFNγ (30) (Fig. 7E), had no effect on NKT-
30   mediated enhancement of T cell responses to the protein. These
data suggest, therefore, that the enhancing effect of NKT cell
stimulation on T cell responses does not require INFγ, but is
CD40L dependent.

35   **Oral administration of antigen together with alpha-GalCer
induces functional CTL.**

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Murine iNKT cells are found with relative abundance in bone marrow, thymus, spleen and liver, while in low numbers in the blood and peripheral lymph nodes. It is likely, therefore, that this distribution of cells will determine the efficacy of

5   administration of iNKT cell agonists by different routes. As vaccine administration via mucosal surfaces is generally seen as a preferred option in terms of likely patient uptake and ease of administration we examined the possibility of inducing immune responses by the oral route. Proteolytic activity in

10   the digestive tract meant that the dose of OVA used was necessarily high, with 30 mg administered per animal. In many reported instances oral administration of protein at this dose has lead to the induction of T cell tolerance, rather than priming, highlighting a potential pitfall of administration of

15   a vaccine by this route. Using tetramers to monitor immune responses in the blood 7 d after antigens were administered, small populations of $OVA_{257-264}$-specific T cells were detected in some animals treated with protein in vehicle (Figure 8a). Subsequent analysis of cytotoxic capacity of the induced

20   populations in vivo by VITAL assay indicated insignificant cytotoxic function (Figure 8b), reflecting either the small size of the induced populations, or perhaps a blunted phenotype of the antigen-specific cells. In striking contrast, easily detected populations of $OVA_{257-264}$-specific cells,

25   representing an average of 0.8% of $CD8^+$ cells, were induced when protein was administered orally together with alpha-GalCer. These populations had cytotoxic capacity, inducing up to 60% lysis of injected targets loaded with 5 μM $OVA_{257-264}$ peptideand could be restimulated in vivo by either vaccinia

30   OVA or OVA in Freund adjuvant   (data not shown). These data suggest that iNKT activation has a very powerful adjuvant effect capable of eliciting effective CTL by oral administration, a route generally considered unfavorable to stimulation of potent effector cells.

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**The enhancing effect of NKT cell stimulation is increased by
stimulation of TLR4 on DC**

The enhanced immune response observed after stimulating NKT
cells can be significantly further enhanced by stimulating
5   simultaneously the TLR4, which is expressed on DC. Stimulation
of NKT cells, via either alphagal Cer or TCR stimulation leads
to the maturation of DC upon the interaction of CD40L
(expressed by activated NKT cells) and CD40 (expressed by DC).
Stimulation of TLR4 (expressed by DC) has a synergistic effect
10   with the CD40 dependent stimulation pathway and results in a
much stronger immune response (Fig. 9). TLR4 binds LPS and a
derivative of LPS "MPL", which is not toxic and can be
injected into patients.

15   The experiment shown in Fig. 9 was carried out by
incorporating alphaGalcer + ovalblumin + MPL into liposomes.
The liposomes were injected i.v. into mice and 7 days later
frequency of OVA specific CTL was measured by ex-vivo tetramer
staining. Liposomes containing either ovalbumin and MPL or
20   ovalbumin and alphaGalceramide were used a control. The
results of these experiments show a very significant
enhancement of the immune response in mice immunized with
liposomes containing alphaGalcer + ovalblumin + MPL (i.e. OVA:
0.1% of tetramer+ cells; OVA + alphaGalcer: 2% of tetramer+
25   cells; OVA+alphaGalcer+MPL: 13% of tetramer+ cells.
We extended these results by demonstrating that co-injection
of alphaGalceramide and MPL can mature DC more efficiently
than either alphaGalceramide or MPL alone. Similar results
were obtained using alphaGalceramide + the TLR 9 ligand Cpg,
30   alphaGalceramide + the TLR5 ligand flagellin and
alphaGalceramide + the TLR 3 ligand poly I:C (Fig. 10).

To assess whether the synergistic effect of alphaGalceramide +
TLR ligands could be extended to other compounds capable of
35   binding to CD1d molecules and activating NKT cells,
experiments were carried out using the compound OCH (30),

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which has a shorter sphyngosine chain as compared to
alphaGalCer. Results of the experiments shown in Fig. 12 and
13 demonstrate that co-injection of OCH + MPL results in
enhanced maturation of DC in vivo and enhanced frequency of
5   OVA specific CTL.

Similar results were obtained in vitro demonstrating the
ability of DC matured in vivo by OCH + MPL to stimulate in
vitro proliferation and gamma secretion of splenocytes from
10   mice transgenic for the $NP_{366-374}$ influenza NP peptide (Fig. 14).

**Administration of α-GalCer together with soluble antigen
provides potent anti-tumour responses**
The therapeutic potential of initiating immune responses with
15   soluble antigen and concomitant activation of NKT cells was
investigated. The tumour cell line EG7.OVA, a derivative of
the murine thymoma EL4 transfected with chicken ovalbumin cDNA
(33), grows progressively when injected s.c. into C57BL/6
mice. When recipients were treated with ovalbumin together
20   with vehicle 11 days prior to tumour challenge, no notable
anti-tumour response was initiated, and tumour engraftment and
progression was observed. This was despite a weak, but
measurable $OVA_{257-264}$-specific $CD8^+$ T cell response in the blood
at day 7 post ovalbumin injection (data not shown). In
25   contrast, when the recipients were treated with ovalbumin
together with α-GalCer prior to tumour challenge, significant
resistance to tumour progression was initated, with the
majority of animals remaining tumour free in excess of 40 days
after tumour challenge. This experiment highlights the
30   potential for immunotherapeutic strategies designed upon the
knowledge that NKT stimulation can significantly potentiate T
cell responses to soluble antigen.

Discussion
35   The ability of DC to promote or suppress adaptive responses to
antigen may ultimately be defined by the signals received by

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immature DC as they transit from peripheral sites to the
draining lymph nodes, where adaptive responses are dictated.
In this report we show that NKT cells responding to
glycolipids presented by monomorphic CD1d molecules can
5   significantly regulate T cell responses to soluble antigen in
vivo, and that this modulation is through direct interaction
with DC. NKT cells therefore represent an abundant source of
the T cell derived factors required for DC activation, and
that these cells can potentially be rapidly mobilized in the
10   generation of T cell mediated responses. Crucially, NKT cells
can provide an early source of CD40L signals that are known to
be required for Th1-biased responses, and CTL induction.

Activation of NKT cells by administration of α-GalCer together
15   with intravenous delivery of soluble ovalbumin protein results
in considerable enhancement of ovalbumin-specific T cell
responses, including CTL responses reliant upon cross
presentation. Analysis of splenic DC isolated from animals
injected with α-GalCer indicated that NKT activation induces
20   upregulated expression of co-stimulatory molecules reflecting
a more mature DC status. Thus, appropriately timed
administration of protein relative to α-GalCer allowed
processed protein to be presented on DC with enhanced
stimulatory function. A cognate interaction between NKT cell
25   and DC was critical to the enhancement of T cell responses
observed as the potentiating effect of α-GalCer on injection
of peptide-loaded BM-derived DC occurred only when the
glycolipid was presented on the same DC as the peptide. These
experiments were undertaken with DC that had been loaded with
30   a minimal MHC class I-binding peptide, rather than whole
protein, so that $CD8^+$ T cell responses could be interpreted
without any potentially confounding effects of NKT cells on
protein processing and presentation.

35   The effect of NKT interaction on DC function proved to be a
more powerful stimulus to $CD8^+$ T cell responses than induction

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of conventional $CD4^+$ T cell help provided by loading both MHC class II-binding and NHC class I-binding peptides onto the injected DC. This is presumably due to the greater number of NKT cells in the lymphoid tissues relative to naïve antigen-specific $CD4^+$ T cells, and hence an improved probability of interacting with injected DC. DC matured with LPS prior to injection also enhanced the peptide-specific $CD8^+$ T cell responses over those induced with non-matured DC, although this enhancement could not be improved upon with NKT stimulation using α-GalCer. However, the enhancement of T-cell response seen with α-GalCer in vivo was increased by the LPS derivative MPL.

Efficient cross priming of $CD8^+$ T cells requires uptake of antigen by immature DC together with exposure to effective maturation stimuli. The most important stimuli in this regard are thought to be CD40L signals, generally presumed to come from $CD4^+$ T helper cells (8-10). Schulz et al. (41) have reported that effective CD40 triggering of DC, as measured by IL-12 induction, requires initial microbial priming of the DC. In our system, where intravenous injection of soluble ovalbumin alone induced cross-priming, it is most likely that low levels of endotoxin in the commercial source of ovalbumin used was providing such a microbial signal. This signal could then combine with conventional $CD4^+$ T cell-mediated help to induce priming of $CD8^+$ responses. The fact that all cross priming was abrogated in $CD40L^{-/-}$ animals highlights the importance of integrating microbial and CD40-mediated signals in this system. Importantly, however, cross-priming in CD40 proficient animals was always significantly enhanced when α-GalCer was administered together with ovalbumin, most likely reflecting a greater supply of CD40L signals from stimulated NKT cells. Thus, while functional CTL with restimulatory capacity could be induced with ovalbumin alone, a dramatic increase in the quantity of CTL (5-10 times in number) was observed when NKT were concomitantly activated with α-GalCer.

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Furthermore, only CTL induced in the presence of α-GalCer
provided resistance to challenge with ovalbumin⁺ tumours. Thus,
activated NKT cells can provide a potent CD40L-mediated boost
to an otherwise weakly cross-presented response, providing
5   functionally superior CTL.

It is likely that a number of reciprocal exchanges between NKT
cell and DC will ultimately determine the quality of an
adaptive response. Initial engagement between NKT and APC
10   bearing glycolipid ligand leads to release of a burst of IL-4
by NKT cells, which can be achieved in the absence of CD40
signalling ((42) and Fig. 7). This initial engagement may then
lead to increased expression of CD40L on the NKT cell surface
and subsequent interaction with DC expressing CD40. As a
15   consequence, DC will be induced to release IL-12, which, in
turn, stimulates NKT cells to release IFNγ. It has been
suggested that IFNγ produced by NKT cells can induce
upregulation of IL-12 receptors on NKT cells in an autocrine
manner (14). These cells therefore become more sensitive to
20   IL-12, resulting in further IFNγ production. It is also
possible that IFNγ can, in turn, feed back on the DC causing
more release of IL-12, as has been reported for human DC (43).
Other studies have highlighted the events subsequent from this
point, namely the activation of NK cells and release of
25   further IFNγ (44). The inventors demonstrate that a second
outcome of this interaction in which the CD40-mediated
activation of DC leads to upregulation of T cell stimulatory
machinery, including co-stimulatory molecules, and a greatly
enhanced capacity to stimulate naïve T cells reactive to
30   foreign antigen.

If this cycle of events is blocked as a result of inability to
stimulate through CD40, as was the case in CD40L⁻/⁻ recipients,
NKT cells could still respond to α-GalCer by producing IL-4
35   (Fig. 7B), but the activation of DC could not take place. On
the other hand, the induction of IFNγl, an event that occurs

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subsequent to CD40 signalling, is not crucial to the
enhancement of T cell responses to soluble antigen, as
potentiation of CD8^{+} T cell responses was still observed in
IFNγR^{-/-} mice (Fig. 7C) and also when NKT were stimulated with

5   an analogue of α-GalCer with reduced capacity to induce IFNγ
       (Fig. 7D). These data imply that it is the cognate interaction
       between NKT cells and DC that is dictating enhancement of T
       cell responses to soluble antigen, and not a potentiating
       effect of IFNγ on DC function.

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       Studies have shown that α-GalCer can stimulate human Vα24/Vβ11
       cells restricted by CD1d. Activated Vα24/Vβ11 NKT cells can
       exert cytotoxic activity against a number of human tumours in
       vitro, regardless of histological origin (46). Animal studies

15   have also highlighted the utility of this glycolipid in the
       induction of anti-tumour responses. However, these latter
       studies have suggested that direct NKT cell-mediated
       cytoxixicty does not play a significant role in responses to
       tumours in vivo (47). In fact it is the NKT cell-mediated

20   activation of NK cells, and rapid induction of IFNγ, that
       exerts the anti-tumour activity (48), with the anti-angiogenic
       property of IFNγ playing a key role (49). It has been reported
       that some anti-tumour CTL activity may also be invoked through
       the NKT activation with α-GalCer (50, 51). This activity may

25   be dependent upon NKT-mediated activation of DC that have
       acquired unique tumour antigens. While this may happen to a
       limited degree in tumour-bearing hosts treated with α-GalCer
       alone, a more successful anti-tumour response would be induced
       if concomitant presentation of tumour antigen and α-GalCer was

30   coordinated by injection of unique tumour proteins with the
       glycolipid. In the experiments described herein, resistance to
       challenge with ovalbumin[+] tumours was induced in such a fashion
       (Fig. 9). Therapy of this type could potentially be used in
       all cancer patients, regardless of MHC haplotype, as the

35   adjuvant capacity of NKT cells is dependent upon presentation
       of α-GalCer by monomorphic CD1d molecules.

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